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Catechin-based extract optimization obtained from *Arbutus unedo* L. fruits using maceration/microwave/ultrasound extraction techniques

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ABSTRACT

This study compares three extraction techniques (maceration, microwave and ultrasound) for catechin recover from *Arbutus unedo* fruit extracts. To obtain the conditions that maximize catechin extraction yield, a response surface methodology was applied using a 3-level full factorial Box–Behnken design in which the processing time (t), temperature (T), ultrasonic power (W) and ethanol percentage (Et%) were the relevant independent variables with the response (catechin content, mg/g dw) measured by HPLC-PDA. A fixed solid/solvent ratio of 50 g/L was used in all techniques. Maceration and microwave extractions were found to be the most effective methods, capable of yielding 1.38 ± 0.1 and 1.70 ± 0.3 mg/g dw of catechin, respectively at the optimal extraction conditions. The optimal conditions for maceration were 93.2 ± 3.7 min, 79.6 ± 5.2 °C and 23.1 ± 3.7% of ethanol, while for the microwave extraction were 42.2 ± 4.1 min, 137.1 ± 8.1 °C and 12.1 ± 1.1% of ethanol. Comparatively with maceration, the microwave system was a faster solution, conducting to slightly higher catechin yields, but using higher temperatures to reach similar values. The ultrasound method was the least effective solution, yielding 0.71 ± 0.1 mg/g dw of catechin at 42.4 ± 3.6 min, 314.9 ± 21.2 W and 40.3 ± 3.8% ethanol. The results highlight the potential of using *A. unedo* fruits bio-residues as a productive source of catechin.

1. Introduction

The small tree of *Arbutus unedo* L. (known as strawberry tree), belonging to the Ericaceae family, is a native species from the Mediterranean region. It produces an edible reddish sweet and tasty berry which is, when fully matured, rich in nutritional properties and has several medicinal effects, as astringent, diuretic and antiseptic properties (Ziyyat et al., 2002). Some authors have reported the presence of phenolic compounds in *A. unedo* fruits (Alarcão-E-Silva et al., 2001; Fortalezas et al., 2010; Guimarães et al., 2013; Pabuçcuoğlu et al., 2003; Pawlowska et al., 2006; Ruiz-Rodríguez et al., 2011), in particular catechins (monomeric flavan-3-ols and procyanidins polymeric flavan-3-ols) (Gadkari and Balaraman, 2015; Guimarães et al., 2013; Pallauf et al., 2008). Catechins are flavan-3-ols that have attracted attention particularly due to their relative high antioxidant capacity (Aron and Kennedy, 2008). Several studies pointed out the interest of using catechins for health benefits, such as cancer prevention and plasma oxidation, as well as obesity control (Higdon and Frei, 2003; Hirasawa and Takada, 2004; Lotito and Fraga, 1998; Nagao et al., 2009). However, flavan-3-ols, and particularly catechin, are susceptible to degradation under various conditions, highlighting the importance of optimizing the extraction conditions to maximize the yield in these compounds (Ananingsih et al., 2013).

A broad spectrum of solid-liquid procedures is available for the extraction and isolation of new functional ingredients (Galanakis, 2012). However, some techniques comprise disadvantages requiring long times, large solvent consumption and leading to thermal degradation of phenolic compounds (Alonso-Salces et al., 2001; Dai and Mumper, 2010; Ince et al., 2013). The choice of the extraction

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solvent has also impact in extraction yield. Catechins are generally extracted with water, polar organic solvents, and aqueous organic solvent mixtures (Piñeiro et al., 2004; Vuong et al., 2010). Maceration extraction (ME) is a conventional method frequently used in the extraction of bioactive compounds. The procedure consists in stirring the sample in a solvent for a certain period of time and at a specific temperature. It is a simple technique, but very often requires long time periods and high temperatures. To overcome these drawbacks, alternative extraction methods are being proposed, such as microwave and ultrasound based techniques (Galanakis, 2013). Ultrasound-assisted extraction (UAE) is a technique that is increasingly used in chemical and food industries (Chemat et al., 2017). This technique has advantages such as being faster than conventional extraction methodologies, it is energetically less demanding and often permits the reduction in the solvent consumption. Adding to this, it generally results in extracts with improved purity and yield. Such advantages result from the process principle that is based on cavitation effects causing the rupture of plant cell walls, thus increasing the contact area between the solid and solvent (Ghasemzadeh et al., 2014; Herrera and Luque De Castro, 2004; Pingret et al., 2012). On the other hand, microwave-assisted extraction (MAE) is a process that facilitates the partition of the sample compounds into the solvent, decreasing the extraction time and temperature, and increasing the process efficiency using lower amounts of solvent (Li et al., 2013a). This method has been employed in the extraction of medicinal herbal compounds (Chen et al., 2008; Dai and Mumper, 2010; Proestos and Komaitis, 2008).

ME, MAE and UAE depend on several process variables whose values cannot be generalized for all matrices due to their specificity in terms of composition and target compounds (Jacotet-Navarro et al., 2016). Thus optimization of process variables is needed to select the best conditions to ensure a maximum yield, minimum time consumption, energy and solvent, obtaining the maximum benefit from the technique (Li et al., 2013b). Traditionally, optimization is achieved by monitoring the influence of one factor at a time. However, by using the response surface methodology (RSM), optimization is done simultaneously and in a multivariable form; the interaction effects between the factors can be assessed allowing a much more precise identification of the optimal conditions. The RSM, by means of mathematical equations, can describe the behaviour of the various variables and forecast the results for the system (Bezerra et al., 2008; Ferreira et al., 2007; Kalil and Maugeri, 2000).

The present study aims to optimize catechin extraction yield from *A. unedo* fruits to be considered for food, pharmaceutical and cosmetic industries. Different extraction methodologies such as ME, MAE and UAE were studied and compared. The joint effect of the relevant variables for each technique, to maximize catechin extraction yield, was described through RSM, contributing to the understanding of the real potential of catechin obtainment from *A. unedo* for industrial applications.

2. Materials and methods

2.1. Source material

The fruits of *Arbutus unedo* L. (strawberry tree) from Ericaceae were gathered in the Natural Park of Montesinho territory, in Trás-os-Montes, North-eastern Portugal. The botanical identification was confirmed by Dr. Ana Maria Carvalho (School of Agriculture, Polytechnic Institute of Bragança, Trás-os-Montes, Portugal) according with a previous report of the authors (Guimarães et al., 2013). The fruits were lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) and stored in a deep-freezer at −20 °C for subsequent analyses.

2.2. Standards and reagents

Formic acid and acetonitrile of HPLC grade from Fisher Scientific (Lisbon, Portugal) were used. Catechin standard was purchased from Sigma (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). All other chemicals and solvents were of analytical grade and purchased from common suppliers.

2.3. Extraction techniques

From a combination of single variable preliminary experiments, previous extractions performed in our laboratory and bibliographic survey, the relevant variables and the appropriate tested ranges for each of the studied extraction techniques were selected and tested. A detailed description of the study ranges for the selected variables in each technique (RSM design) are described in Table A1 (Supplemental material section). The solid/solvent ratio was kept constant (50 g/L) for all techniques. The used solvent was an ethanol/water mixture characterized in terms of ethanol content.

2.3.1. Maceration extraction (ME)

The lyophilized powdered fruit samples (1 g) were placed in a beaker with 20 mL of solvent in order to obtain the desired solid/liquid ratio (50 g/L). The beaker was placed in a thermostated water bath under continuous electro-magnetic stirring for the required time period. The variables and ranges tested were: time (t or X_1, 20–150 min), temperature (T or X_2, 20–90 °C) and ethanol percentage (S or X_3, 0–100%).

2.3.2. Microwave-assisted extraction (MAE)

MAE process was performed using a Biotage Initiator Microwave (Biotage® Initiator$^+$, Uppsala, Sweden) using closed vessels. The lyophilized powdered samples (1 g) were extracted with 20 mL of solvent (solid/solvent ratio 50 g/L). In microwave systems the pressure and T are correlated and the applied power linked to the needed t to reach the selected T or pressure. In consequence, T was selected as the main variable and the microwave power was set to 400 W. Under the selected conditions, the needed t to reach the selected T was always less than 20 s thus guarantying a fast heating process (this time can be neglected face to the studied extraction time range). Therefore, the final variables and ranges tested were t (X_1, 1.6–45 min), T (X_2, 50–145 °C) and S (X_3, 0–100%).

2.3.3. Ultrasound-assisted extraction (UAE)

The UAE was carried out in an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA). The lyophilized powdered samples (2.5 g) were extracted with 50 mL (solid/solvent ratio 50 g/L) by the ultrasonic device at different times (t or X_1, 5 to 55 min) and at different ultrasound power ranges (P or X_2, 100–400 W) according to an ethanol content (S or X_3, 0–100%) while temperature was monitored in order to be below 30–35 °C.

2.4. Extract purification

The collected extracts were filtered through a Whatman paper filter n° 4. Then, the filtered material was dried at 40 °C in a rotary evaporator Büchi R-210 (Flawil, Switzerland). For purification, a C_{18} SepPak® Vac 3 cc cartridge (Phenomenex) was used. After being activated with ethanol followed by water; sugars and more polar substances were removed by passing the column with 10–20 mL of water. Then the purified extract was further eluted with 10–15 mL

of ethanol. The purified extract was dried at 40° C to remove ethanol.

2.5. Catechin quantification by HPLC-PDA

The samples obtained during the extraction optimization studies were analysed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan) with a quaternary pump and a photodiode array detector (PDA) coupled to an LC solution software data-processing station. Separation was achieved using a Waters Spherisorb S3 ODS-2C_{18}, (3 μm, 4.6 mm × 150 mm) column operating at 35 °C. The used mobile phase was a mixture of formic acid in water 0.1% (A) and100% of acetonitrile (B), and the established elution gradient was as follows: 15% B for 5 min, 15% B to 20% B over 5 min, 20–25% B over 10 min, 25–35% B over 10 min, 35–50% B for 10 min, and column re-equilibration (15 min), using a flow rate of 0.5 mL/min. Detection was carried out in the PDA at 280 nm as preferred wavelength. Catechin was identified by comparing its UV spectra and retention times with the ones of a commercial standard as reported previously (Guimarães et al., 2013). The quantitative analysis was performed using a calibration curve based on catechin (y = 66243x-343411; R^2 = 0.999). Results were expressed in mg of catechin per g of dry fruit weight (mg/g dw).

2.6. Response surface methodology

2.6.1. Experimental design

For each extraction technique three variables were selected as the relevant ones. Those variables were studied in conjunction with a structured experimental design criteria (Box and Hunter, 1957) using a response surface methodology. Initially, three RSM variables were applied for each technique to optimize the extracting conditions. If the tested experimental range failed to provide a global optimum in any of the three variables, a relative optimum within the tested range was attempted through another RSM design involving the unresolved variable combined with the other relevant variables of the extraction system in a complementary two variables RSM design. Therefore, for complex scenarios, the two different experimental designs used for the optimization of the extraction conditions in each tested technique were as follows:

a) For the analysis of three variables ($X_{1\text{-}3}$): a *circumscribed central composite design* (CCCD) was used. In this design the experimental points are generated on a sphere around the centre point. This design requires 5 levels for each factor and 3 replicates per coordinate.
b) For the analysis of two variables ($X_{1\text{-}2}$): a *full factorial design* (FFD) with three replicates per condition was used. The structure of a FFD implies that all combinations of three values, for each factor, are studied (minimum, mean and maximum).

For both RSM design, the centre point is assumed as a value close to the optimum position for the response, being repeated in order to maximize the prediction precision (Box et al., 2005). Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses. A detailed description of the mathematical expressions to calculate the design distribution and to decode and code the tested variable's ranges can be found in the Appendix section.

2.6.2. Mathematical modelling

Independently of the RSM used (two or three variables) the model for the analysis of the produced responses follows this second-order polynomial equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (1)$$

where Y is the dependent variable (response variable) to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficients of quadratic effect and n is the number of variables. Although the statistical consistent model parameters obtained are empirical and cannot be associated with a mechanistic meaning, they are useful to predict the results of untested operation conditions (Pinela et al., 2016). The sign of the effect marks the response performance. In this way, when a factor has a positive effect, the response is higher at the high level and when a factor has a negative effect, the response is lower at the high level. The higher the absolute value of a coefficient, the more important the weight of the corresponding variable (Heleno et al., 2016).

2.6.3. Procedure to optimize the variables to a maximum response

For optimization of catechin extraction, a maximized process of the model produced responses was achieved, using a simple method tool to solve non-linear problems (Heleno et al., 2016; Pinela et al., 2016). Limitations were made to the variable coded values to avoid unnatural conditions (*i.e.*, times lower than 0).

2.7. Numerical methods, statistical analysis and graphical illustrations

All fitting procedures, coefficient estimations and statistical calculations were performed using a Microsoft Excel spreadsheet and the presented graphical illustrations were developed in the software DeltaGraph V6. Fitting and statistical analysis of the experimental results, according to the displayed equations, were carried out in four phases:

- *Coefficients determination:* Parametric estimates were obtained by minimization of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-square (quasi-Newton) method provided by the macro *Solver* in *Microsoft Excel* 2003 (Kemmer and Keller, 2010), which allows a quick testing of hypotheses and analysis of its consequences (Murado and Prieto, 2013).
- *Coefficients significance:* Determination of the parametric confidence intervals using the '*SolverAid*' (Prikler, 2009). The model was simplified by excluding the values which were not statistically significant at α = 0.05.
- *Model consistency:* The Fisher F test (α = 0.05) was used to determine whether the constructed models were adequate to describe the observed data (Shi and Tsai, 2002).
- *Other statistical assessment criteria:* For confirmation of the uniformity of the model, the following criteria were applied: a) The 'SolverStat' macro (Comuzzi et al., 2003) which is used for the assessment of uncertainties related to parameter and model predictions; b) R^2 which is interpreted as the proportion of the variability of the dependent variable explained by the model; c) Adjusted coefficients of multiple determination (R^2_{adj}), which is a correction to R^2 taking into account the number of variables used in the model; d) Bias and accuracy factors of all equations were calculated to evaluate the quality of fittings to experimen-

tal data, such as the Mean Squared Error (MSE), the Root Mean Square of the Errors (RMSE) and the Mean Absolute Percentage Error (MAPE); e) the Durbin-Watson coefficient (DW) to test, if the residuals of the model are not auto-correlated; and f) the Analysis of Variance table (ANOVA) to evaluate the explanatory power of the variables.

3. Results and discussion

3.1. Preliminary experiments to select the relevant variables and instrumental parameters to centre their experimental domain previous to the RSM application

Although the existing previous reports dealing with the optimization of catechin extraction from natural matrices (Table 1), no reports could be found describing the conditions of catechin extraction from *Arbutus unedo* L. fruits. In addition, due to the compositional diversity of the material sources described in Table 1, the tested conditions cannot be directly extrapolated for catechin extraction from *A. unedo* fruits. Therefore, to find the conditions that maximize catechin extraction from *Arbutus unedo* L., it is necessary to take into account the variables that affect solid/solvent system techniques behaviour. These variables can be divided into non-intrinsic factors (solvent type, S and solid to liquid ratio) and intrinsic factors (t and T for the ME and MAE systems, and t and P for UAE system). Preliminary tests were examined individually to determine their experimental domain (keeping other ones constant) in order to obtain a proper RSM design by analyzing their general pattern responses.

In consequence, in all extracting systems, the non-intrinsic variables and ranges were selected as follows:

1) The extracting solvent type is a key factor for the separation of the desired compounds. Due to the catechin chemical structure, different solvent mixtures with water were used to maximize extraction yields; mainly, water with methanol, ethanol or acetone different contents (more details in Table 1). Due to green chemistry principles, binary mixtures of ethanol with water were selected as the extraction solvent. In all systems, the ethanol content in the water/ethanol mixture (S) was tested from 0 to 100% and confirmed as impacting significantly the catechin extraction yield and, therefore, selected in the appropriate range.
2) With regard to solid/liquid ratio, the tested range was 1–60 g/L lower values lead to an enhanced extraction yield, but also contribute to a significant waste of solvent. A higher solid/liquid ratio will result in lower catechin extraction yields but in a better rationalization of raw materials consumption. However, lower differences were found, discarding the solid/liquid ratio and selecting the 50 g/L as the value to be used in all tested extraction techniques.

Concerning the intrinsic variables from the ME, MAE and UAE, a literature survey concerning the main ranges, as studied in similar processes (Table 1), was carried out. Although good conclusions can be derived from this report, results may be highly dependent on variations not foreseen in these studies where certain variables that remained constant, together with the variability in the used raw materials to extract catechin, can highly influence the process.

In conclusion, the first approach to optimize the efficiency of the ME, MAE and UAE processes for catechin extraction, was performed by the application of a RSM of three variables in a *CCCD*. Five levels of variation for the independent variables of t (20–120 min), T (20–90 °C), and S (0–100%) for ME and of t (1–20 min), T (50–120 °C), and S (0–100%) for MAE and t (5–55 min), P (100–400 W), and S (0–100%) for UAE were used. A detailed description of the coded and natural values of the selected variables for each technique in the *CCCD* with tree variables is presented in Table A1 (Supplemental material section).

3.2. RSM output for a CCCD with three variables

The results obtained according to the statistical *CCCD* are shown in the first part of Table 2 for each of the computed extraction techniques. After fitting Eq. (1) to the response results of Table 2 using a non-linear least-squares procedure, the estimated parametric values, parametric intervals and numerical statistical criteria were obtained and presented in the first part of Table 3. Those coefficients, which showed effects with coefficient interval values ($\alpha = 0.05$) higher than the parameter value, were consider as non-significant (ns) and were not pondered for the model development.

Therefore, mathematical models were built, obtaining the following second-order polynomial equations according to Eq. (1) for each of the assessed extraction techniques:

$$\text{for ME: } Y_{ME} = 1.1 + 0.05t + 0.24T - 0.23S/L - 0.11t^2 - 0.15T^2 - 0.13S/L^2 - 0.1tT \quad (2)$$

$$\text{for MAE: } Y_{MAE} = 0.66 + 0.09t + 0.08T - 0.04S/L - 0.01t^2 - 0.03T^2 - 0.11S/L^2 + 0.03tT - 0.04tS/L - 0.03TS/L \quad (3)$$

$$\text{for UAE: } Y_{UAE} = 0.69 + 0.02t + 0.02T - 0.8S/L - 0.02t^2 - 0.03T^2 - 0.13S/L^2 \quad (4)$$

The Eqs. (2)–(4) translate the response patterns for each extraction technique showing highly complex sceneries (Table 3). Linear and quadratic effects are found playing an important and significant role in all extracting systems. Regarding the interactive effects, for ME system only the interaction between t & T was significant in a positive mode; for MAE all the variable interactions caused a significant effect (positive for t & T, and negative for t & S and T & S); and for UAE no significant effects were found.

Fig. 1 shows the extraction results in mg/g dw of catechin, which is divided in three columns for each one of the tested techniques. Each column is divided into two subsections (A and B). The subsection A shows the combination of the three-dimensional response surface plots predicted with their respective second order polynomial equation described by Eqs. (2)–(4) as a function of each one of the involved variables. The binary action between variables is presented when the excluded variable is positioned at the centre of the experimental domain (see Table A1). Subsection B illustrates the capability to predict the obtained results and the residual distribution as a function of each one of the considered variables.

In almost all combinatory 3D responses of Fig. 1, the amount of extracted material increases to an optimum value and then decreases as a function of each one of the assessed independent variables. Therefore, in almost all combinations the optimum can be found at one single point along with the response, allowing computing the conditions that lead to the absolute maximum.

By applying a simple procedure considering restrictions to the experimental ranges, optimal conditions are found, as well as the maximal response values (first part of Table 4). For the ME system, the relative optimal (*) or absolute conditions found were at 88.3 ± 31.8 min, 79.2 ± 15.7 °C and 23.1 ± 3.7% ethanol, producing a maximum response value of 1.36 ± 0.5 mg of catechin/g dw. For MAE response, the optimal condition values were at $^{*}18.4 \pm 1.7$ min, $^{*}118.6 \pm 21.3$ °C and 12.1 ± 1.1% ethanol,

Table 1
Bibliographic summary of catechin content from different source materials using different extraction techniques and conditions.

TECHNIQUE APPLIED	SOURCE MATERIAL	PLANT PART	EXTRACTION CONDITIONS					CATECHIN CONTENT	REFERENCE
			Solvent	*Temperature*	*Power or Frequency (kHz or W)*	*pH*	*Time*		
			–	(°C)		–	*(min)*	(mg/g dw)	
ULTRASOUND ASSISTED EXTRACTION	Apple	Pulp	Water	40	25 kHz	–	45 to 90	0.030 to 0.075	Mieszczakowska-Frąc et al. (2015)
	Apple	Pomace	Water	40	150 W	3.8	40	0.15	Pingret et al. (2012)
	Curry	Leaves	Methanol:Water (80:20)	56	145 W	–	20	0.48	Ghasemzadeh et al. (2014)
	Grape	Seeds	Methanol:Water (75:25)	–	–	–	15	0.41	García-Marino et al. (2006)
	Grape	Seeds	Methanol	60	–	–	10	0.23	Piñeiro et al. (2004)
	Grape	Seeds	Methanol:Water (10:90)	30	–	–	30x2	0.65	Palma and Taylor (1999)
	Maritime pine	Plant	Water	40	–	3.8	43	3.5	Meullemiestre et al. (2016)
	Melissa	Leaves	Water	–	150 W	–	20	2.01	Ince et al. (2013)
	Mushroon		Ethanol:Water (60:40)	25	–	–	30	0.1	Zhang et al. (2012)
	Pistachio	Nut	Water	–	35 kHz	–	30	0.05	Garavand et al. (2015)
	Strawberrie	Fruit	Acetone	–	100 W	<3	0.5x3	0.015	Herrera and Luque De Castro (2004)
MACERATION ASSISTED EXTRACTION	Apple	Pulp	Water	40	–	–	45–90	0.040 to 0.050	Mieszczakowska-Frąc et al. (2015)
	Apple	Pomace	Water	40	–	3.8	40	0.115	Pingret et al. (2012)
	Folium eriobotryae	Fruit	Methanol	–	700 W	–	3	12.1	Chen et al. (2008)
	Grape	Seeds	Methanol:Water (4:1)	30	–	–	960	0.7	Palma and Taylor (1999)
	Green tea	Leaves	Water	25 to 80	–	<6	30 to 120	0.600 to 7.100	Vuong et al. (2011)
	Melissa	Leaves	Water	40	–	–	1440	3.45	Ince et al. (2013)
	Mushroon		Ethanol:Water (60:40)	25	–	–	720	0.1019	Zhang et al. (2012)
	Grape	Seeds	Methanol	60	–	–	10	0.27	Piñeiro et al. (2004)
HEAT REFLUX EXTRACTION	*F. eriobotryae*	Fruit	Methanol	80	–	–	3	7.34	Chen et al. (2008)
	Rosemary	Plant	Methanol:Water (60:40)	90	–	–	120	0.019	Proestos and Komaitis (2008)
	Mushroon		Ethanol:Water (60:40)	90	–	–	50	0.1023	Zhang et al. (2012)
MICROWAVE ASSISTED EXTRACTION	Rosemary	Plant	Methanol:Water (60:40)	–	750 W	–	4	0.025	Proestos and Komaitis (2008)
	Pistachio	Nut	Water	–	800 W	–	0.5	0.0467	Garavand et al. (2015)
	Melissa	Leaves	Water	–	407 W	–	5	1.353	(Ince et al. (2013)
	Mushroon		Ethanol:Water (60:40)	110	500 W	–	10	0.1079	Zhang et al. (2012)
PRESSURIZED LIQUID EXTRACTION	Apple	Peel	Methanol	40	–	–	5	0.043	Alonso-Salces et al. (2001)
	Apple	Pulp	Methanol	40	–	–	5	0.018	Alonso-Salces et al. (2001)
	Grape	Seeds	Methanol	130	–	–	10	1.82	Piñeiro et al. (2004)
SUPRECRITICAL EXTRACTION	Grape	Seeds	Methanol:Water (10:90)	55	–	–	60	0.865	Palma and Taylor (1999)
SUBCRITICAL EXTRACTION	Grape	Seeds	Water	50, 100, 150	–	–	30	0.14 to 0.47	García-Marino et al. (2006)

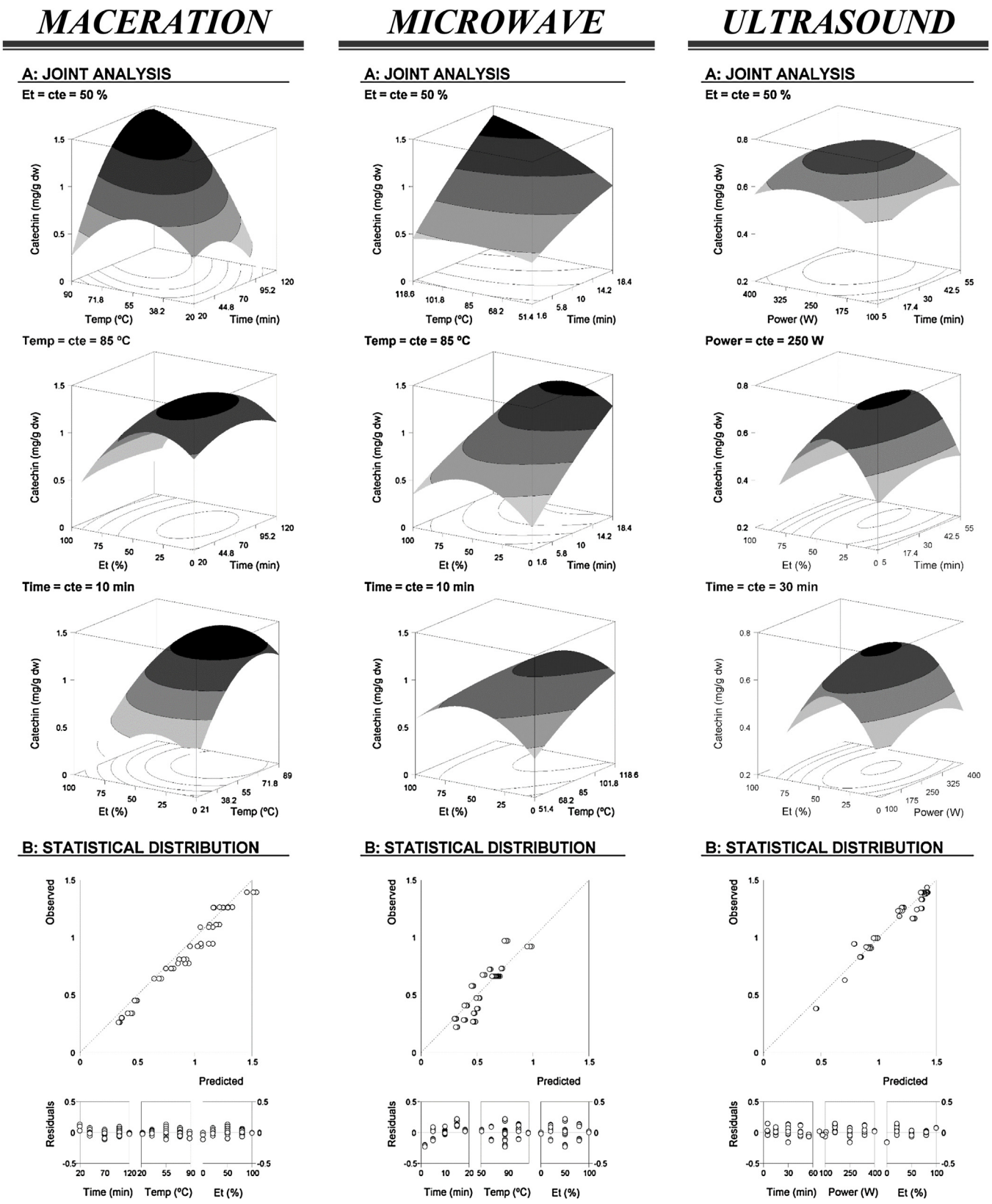

Fig. 1. Shows the graphical results in terms of the extraction behaviour for the CCCD. *Part A*: Shows the joint graphical 3D analysis as a function of each the variables involved. Each of the net surfaces represents the theoretical three-dimensional response surface predicted with the second order polynomial of Eqs. (2)–(4). The binary actions between variables are presented when the excluded variable is positioned at the centre of the experimental domain (Table A1). The statistical design and results are described in Table 2. Estimated parametric values are shown in Table 3. *Part B*: To illustrate the goodness of fit, two basic graphical statistic criteria are used. The first one, the ability to simulate the changes of the response between the predicted and observed data; and the second one, the residual distribution as a function of each of the variables. Note all the differences in the axes scales.

Table 2
Part A shows the RSM results of the CCCD for the first approach for the optimization of the three main variables involved (X_1, X_2 and X_3) in the ME, MAE and UAE. Part B shows the RSM results of the FFD for the optimization of the two variables (X_1 and X_2) involved for the ME and MAE (variables, natural values and ranges in Table A1). Three replicates ($r_{1\text{-}3}$) were performed for each condition for each technique.

VARIABLE CODED VALUES			CATECHIN CONTENT (mg/g dw)								
			MACERATION			MICROWAVE			ULTRASOUND		
X_1	X_2	X_3	r_1	r_2	r_3	r_1	r_2	r_3	r_1	r_2	r_3
A) CIRCUMSCRIBED CENTRAL COMPOSITE DESIGN (CCCD)											
−1	−1	−1	0.84	0.86	0.87	0.31	0.32	0.33	0.66	0.65	0.65
1	−1	−1	0.74	0.76	0.77	0.55	0.56	0.57	0.67	0.67	0.67
−1	1	−1	1.12	1.15	1.16	0.50	0.50	0.51	0.68	0.69	0.69
1	1	−1	1.32	1.35	1.36	0.95	0.98	0.99	0.69	0.69	0.69
−1	−1	1	0.38	0.39	0.40	0.38	0.39	0.40	0.42	0.42	0.42
1	−1	1	0.30	0.30	0.30	0.45	0.46	0.47	0.45	0.47	0.46
−1	1	1	0.65	0.67	0.68	0.47	0.47	0.48	0.47	0.45	0.46
1	1	1	0.94	0.97	0.99	0.61	0.61	0.62	0.48	0.50	0.49
−1.68	0	0	0.81	0.84	0.85	0.49	0.46	0.47	0.61	0.60	0.60
1.68	0	0	0.99	1.03	1.04	0.74	0.76	0.77	0.71	0.71	0.71
0	−1.68	0	0.40	0.41	0.41	0.49	0.52	0.53	0.58	0.60	0.59
0	1.68	0	1.18	1.21	1.23	0.72	0.72	0.73	0.70	0.70	0.70
0	0	−1.68	1.14	1.17	1.19	0.39	0.41	0.41	0.39	0.40	0.39
0	0	1.68	0.39	0.39	0.4	0.30	0.31	0.32	0.23	0.23	0.23
0	0	0	1.17	1.21	1.23	0.63	0.64	0.65	0.69	0.69	0.69
0	0	0	1.17	1.21	1.23	0.65	0.67	0.68	0.68	0.68	0.68
0	0	0	1.17	1.21	1.23	0.66	0.70	0.71	0.71	0.71	0.71
0	0	0	1.17	1.21	1.23	0.66	0.68	0.69	0.69	0.70	0.70
0	0	0	1.17	1.21	1.23	0.67	0.68	0.69	0.71	0.71	0.71
0	0	0	1.17	1.21	1.23	0.67	0.68	0.69	0.69	0.70	0.70
B) FULL FACTORIAL DESIGN (FFD)											
−1	−1	–	1.16	1.16	1.16	0.93	0.94	0.94	–	–	–
0	−1	–	1.24	1.25	1.25	1.35	1.36	1.37	–	–	–
1	−1	–	0.83	0.83	0.83	1.51	1.52	1.53	–	–	–
−1	0	–	1.30	1.30	1.30	1.22	1.23	1.24	–	–	–
0	0	–	1.40	1.40	1.40	1.60	1.61	1.62	–	–	–
1	0	–	1.00	1.01	1.00	1.72	1.74	1.75	–	–	–
−1	1	–	1.27	1.27	1.27	1.19	1.20	1.20	–	–	–
−1	−1	–	1.38	1.38	1.38	1.53	1.54	1.54	–	–	–

Table 3
Parametric results of the second-order polynomial equation (Eq. (1)) for each of the extracting technique assessed according to the CCCD with 5 range levels (part A) and FFD with 3 range levels (part B). The parametric subscript 1, 2 and 3 stands for the variables involved *t*, *T* and *S*, respectively. Analysis of significance of the parameters (α=0.05) are presented in natural values. Additionally, the statistical information of the fitting procedure to the model is presented.

COEFFICIENTS		RESPONSES				
		CENTRAL COMPOSITE DESIGN			*FULL FACTORIAL DESIGN*	
		MACERATION	*MICROWAVE*	*ULTRASOUND*	*MACERATION*	*MICROWAVE*
Fitting coefficients obtained						
Intercept	b_0	1.126 ± 0.10	0.668 ± 0.37	0.6960.016	1.351 ± 0.02	1.613 ± 0.02
Linear effect	b_1	0.051 ± 0.01	0.091 ± 0.02	0.0190.01	0.076 ± 0.01	0.089 ± 0.01
	b_2	0.241 ± 0.06	0.076 ± 0.02	0.0220.01	−0.114 ± 0.01	0.219 ± 0.01
	b_3	−0.235 ± 0.06	−0.034 ± 0.02	−0.0840.01		
Quadratic effect	b_{11}	−0.112 ± 0.03	−0.009 ± 0.00	−0.0170.01	−0.079 ± 0.01	−0.184 ± 0.02
	b_{22}	−0.154 ± 0.06	−0.025 ± 0.00	−0.030.01	−0.227 ± 0.01	−0.15 ± 0.02
	b_{33}	−0.13 ± 0.06	−0.111 ± 0.02	−0.1290.01		
Interactive effect	b_{12}	0.09 ± 0.08	0.025 ± 0.02	*ns*	0.017 ± 0.01	−0.041 ± 0.01
	b_{13}	*ns*	−0.042 ± 0.02	*ns*		
	b_{23}	*ns*	−0.025 ± 0.02	*ns*		
Statistical information of the fitting analysis						
Obs		60	60	60	27	27
df		51	49	52	20	20
R^2		0.967	0.9655	0.9479	0.986	0.9879
R^2adj		0.9326	0.9593	0.9386	0.9625	0.9631
MEC		0.131	0.0311	0.022	0.0864	0.049
RMSE		0.362	0.1762	0.1483	0.294	0.2213
MAPE		2.0099	4.0771	4.6536	0.3814	0.0917
DW		1.0598	2.5446	1.6565	0.236	2.9929

ns: non significant coefficient; *Obs*: Number of observations; *df*: Number of degrees of freedom; R^2: Correlation coefficient; R^2adj: The adjusted determination coefficient for the model; *MSE*: The Mean Square of the Error; *RMSE*: The Root Mean Square of the Errors; *MAPE*: The Mean Absolute Percentage Error; and *DW*: The Durbin-Watson statistic.

producing a maximum response of 0.97 ± 0.2 mg catechin/g dw. For UAE, the optimal conditions were found at 42.4 ± 4.1 min, 314.9 ± 21.2 W and 40.3 ± 3.8% ethanol obtaining a maximum response of 0.71 ± 0.1 mg catechin/g dw.

Table 4
Variable conditions in natural values that lead to optimal response values for the first approximation RSM using a CCCD and for the second using a FFD for each of the extracting techniques assessed.

CRITERIA	OPTIMAL VARIABLE CONDITIONS			OPTIMUM RESPONSE	
	X_1: t (min)	X_2: T (°C) or P(W)	X_3: S (%)		
Individual optimal variable conditions for the CCCD:					
Maceration	88.3 ± 31.8	79.2 ± 15.7*	23.1 ± 3.7	1.36 ± 0.5	*mg/g dw*
Microwave	18.4 ± 1.7*	118.6 ± 21.3*	12.1 ± 1.1	0.97 ± 0.2	*mg/g dw*
Ultrasound	42.4 ± 4.1	314.9 ± 21.2	40.3 ± 3.8	0.71 ± 0.1	*mg/g dw*
Individual optimal variable conditions for the FFD:					
Maceration	93.2 ± 3.7	79.6 ± 5.2	–	1.38 ± 0.1	*mg/g dw*
Microwave	42.2 ± 4.1	137.1 ± 8.1	–	1.70 ± 0.3	*mg/g dw*
Global optimal variable conditions for the combination of the CCCD and FFD responses:					
Maceration	93.2 ± 3.7	79.6 ± 5.2	23.1 ± 3.7	1.38 ± 0.1	*mg/g dw*
Microwave	42.2 ± 4.1	137.1 ± 8.1	12.1 ± 1.1	1.70 ± 0.3	*mg/g dw*
Ultrasound	42.4 ± 3.6	314.9 ± 21.2	40.3 ± 3.8	0.71 ± 0.1	*mg/g dw*

Although the CCCD was based on preliminary tests and bibliographic results, in the produced responses, it was not possible to find the optimal conditions for all variables in the tested extraction techniques. The main reason is due to the fact that the experiments were conducted at one factor of the time analysis and the obtained patterns do not take into account the interactive effects. Only experimental designs based on multivariable analysis (such as the RSM) can produce patterns that integrate the interactions between the variables. The positive interactions between the *t* & *T* in ME and MAE systems produced an additional effect since the responses were not either conclusive enough (ME case) or absolutely optimized (MAE case) within the tested variable's range, finding large confidence intervals for some optimal values (ME case) or relative optimum conditions (MAE case) for the variables *t* and *T*. The lack of a clear absolute optimum in the provided solution, forces the acceptance of one of the following solutions: 1) an unreliable optimum and/or a relative optimum; 2) to use the predicting absolute optimum values of the developed mathematical model; or 3) to re-design a second RSM around the ranges that seem to be the optimal ones in order to find the experimental values that would help to find the absolute optimum of these variables in which the first optimization approach failed. In this study, solution (3) was chosen and further experiments were performed using a RSM based in a *FFD* for the specific analysis of the interaction of *t* and *T* variables in ME and MAE systems.

3.3. Final optimization of ME and MAE using a RSM based in FFD with the t and T variables

The variables range of *t* and *T* for the *FFD* were expanded according to the *CCCD* results (experimental domain in second part of Table A1, supplemental material). The obtained results, according to the statistical *FFD* for ME and MAE, are shown in the second part of Table 2 for each of the computed extraction technique. Identically to the previous RSM approach, Eq. (1) was used to fit the results of Table 2 using a non-linear least-squares procedure. The estimated parametric values, parametric intervals and numerical statistical criteria were obtained and presented in the second part of Table 3. Mathematical models were built, obtaining the following second-order polynomial equations according to Eq. (1) for each one of the assessed extraction technique:

$$\text{for ME: } Y_{ME} = 1.35 + 0.08t - 0.11T - 0.08t^2 - 0.23T^2 + 0.08tT \quad (5)$$

$$\text{for MAE: } Y_{MAE} = 1.6 + 0.09t + 0.2T - 0.18t^2 - 0.15T^2 - 0.04tT \quad (6)$$

Eqs. (5) and (6) complete the response patterns for each extraction technique showing nearly identical sceneries to those previously found for the *CCCD* approach, but covering a more extensive range of the variables, allowing to find the extraction conditions that lead to a reliable absolute optimum for catechin extraction. Linear and quadratic effects were found to play an important and significant role in all extraction systems. Regarding the interactive effects of *t* and *T*, for ME and MAE, significant effects were corroborated in a positive and negative form, respectively.

Fig. 2 shows the catechin extraction results for each one of the tested techniques (ME and MAE). For each technique Fig. 2 is divided into three sections:

- Section A shows the catechin extraction yield (mg/g dw) as a function of *t* and *T* variables. Points (●) represent the obtained experimental results according to the described statistical design. The net surface represents the theoretical three-dimensional response surface predicted with the second order polynomial Eqs. (5) and (6). Estimated parametric values are shown in the second part of Table 3. The binary action between variables is presented when the excluded variable is positioned at the centre of the experimental domain.
- Section B presents two-dimensional representation of the fitting results of Eqs. (5) and (6) (solid line) to the experimental points (□ minimum, ◊ medium and △ maximum variable values) of the combined effect of *t* and *T* on the catechin extraction yield (mg/g dw).
- Section C shows an illustration for the statistical robustness of the reached solution. Two basic graphical criteria are used: the ability to simulate the response changes and the residual distribution as a function of each of one of the variables.

In both techniques (ME and MAE) the *T* and *t* variables significantly affected catechin extraction. Catechin extraction efficiency increased with the increase of *T* and *t* until an absolute optimum from which it decreased. By applying a simple procedure with restrictions to the tested experimental ranges, the optimal condition results can be found, as well as, the maximal catechin yield response values for each technique, being presented in the second part of Table 4. For the ME system, the optimal absolute conditions found were at 93.2 ± 3.7 min and 79.6 ± 5.2 °C (at a constant 24% of ethanol) producing a maximum response value of 1.38 ± 0.1 mg of catechin/g dw. For the MAE response, the optimal condition values were at 42.2 ± 4.1 min and 137.1 ± 8.1 °C (at a constant 12% of ethanol) producing a maximum response of 1.70 ± 0.3 mg of catechin/g dw.

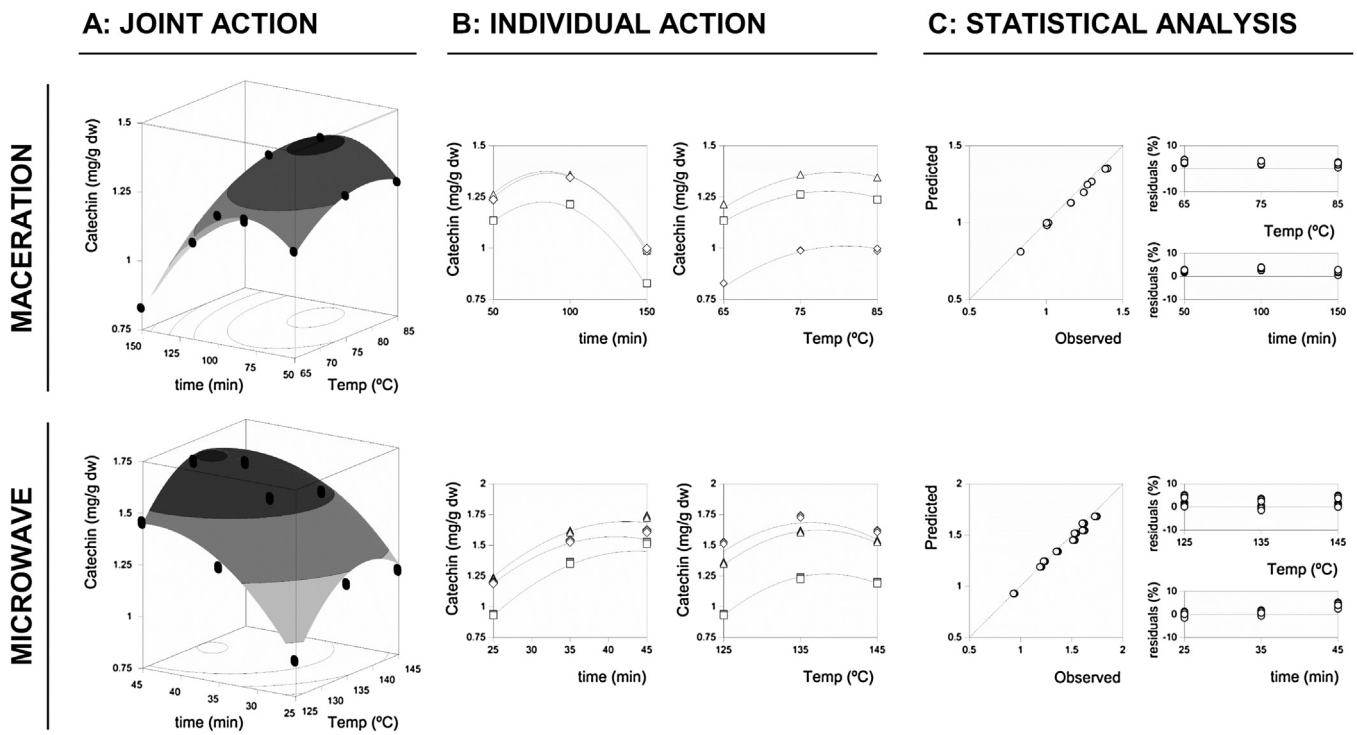

Fig. 2. Shows the final optimization extracting results of ME and MAE techniques in a FFD. A: Catechin extraction yield (mg/g dw) as a function of extracting time (*t*) and temperature (*T*). Points (●) represent the obtained experimental results (second part of Table 2) according to the statistical design described (second part of Table A1). The net surface represents the theoretical three-dimensional response surface predicted with the second order polynomial Eqs. (5) and (6). Estimated parametric values of are shown in second part of Table 3. B: Two-dimensional representation of the fitting results of Eqs. (5) and (6) (solid line) to the experimental points (□ minimum, ◊ medium and △ maximum variable values) of the combined effect of *P* and *t* on catechin extraction yield (mg/g dw). C: To illustrate the statistical description, two basic graphical criteria are used: the ability to simulate the changes of the response and the residual distribution as a function of each of the variables.

3.4. Extraction techniques comparison, numerical optimal conditions that maximize the extraction, statistical analysis and experimental verification of predictive models

ME, MAE and UAE extraction techniques have been optimized and compared concerning the recovery of catechin rich extracts from *A. unedo* fruits. These solid/liquid extraction methods have been applied to the extraction of catechin from different source materials (García-Marino et al., 2006; Ghasemzadeh et al., 2014; Meullemiestre et al., 2016; Mieszczakowska-Frąc et al., 2015; Palma and Taylor, 1999; Pingret et al., 2012; Piñeiro et al., 2004).

Although a scientific survey focusing catechin content in different source materials was conducted, pointed out the use of several extraction techniques, as far as we know, there are no references in the literature describing the optimization of catechin extraction from *A. unedo* fruits. Comparing the obtained yields of the present work with the ones available in literature, *A. unedo* fruits can be considered a suitable source for catechin extraction obtainment (Ananingsih et al., 2013; Gadkari and Balaraman, 2015).

When combining the information produced from the *CCCD* and *FFD* approaches, the complete behaviour of each relevant variable influencing catechin extraction is defined in absolute terms. For all techniques the conditions that lead to the optimal values were re-checked in order to ensure the accuracy of the presented results. Fig. 3 shows the summarized individual 2D responses as a function of the defined variables for ME, MAE and UAE extraction techniques to guide the selection of the most favourable conditions. The line represents the variable response pattern when the others are located at the optimal values presented in the third part of Table 4. The dots (⊙) presented alongside the line highlight the location of the optimal value. Comparing the results of extraction efficiencies among techniques, ME and MAE gave significantly higher values, while UAE extraction generated lower values probably due to degradation flavan-3-ols and particularly catechin as it occurs in other natural compounds (Jacotet-Navarro et al., 2016; Li et al., 2013b). Regarding the extraction time, MAE was the fastest extraction method with ~45 min while ME needed ~95 min. Considering extraction efficiency, maceration give similar results to MAE. UAE was found not adequate catechin extraction due to its low extraction efficiency.

The performed characterization to optimize catechin extraction yield in ME, MAE and UAE with the RSM provides a strong solution that minimizes the errors with a short number of experimental trials as it has benn demonstrated elsewhere (Roselló-Soto et al., 2015; Wong et al., 2015). The multivariable fitting decreases the number of parameters needed to analyze the response leading to better estimations and reducing their interval of confidence.

The lack-off-fit test used to assess the competence of the models showed that the non-significant parameters of both RSM approaches (Table 3) did not statistically improve the reached solution and, in contrast, all significant parameters were highly consistent ($p < 0.01$). This was also verified by the achieved high R^2 and R^2_{adj} values, indicating the percentage of variability explained by the model (Table 3). The distribution of the residuals presented in Fig. 1 and Fig. 2 was arbitrarily around zero and no group of values or autocorrelations were observed. Additionally, the agreement between the experimental and predicted values implies an acceptable explanation of the results obtained by the independent variables used. Therefore, the models developed in Eqs. (2)–(6), either for the *CCCD* or *FFD*, are completely functional and adequate to be used for prediction and process optimization.

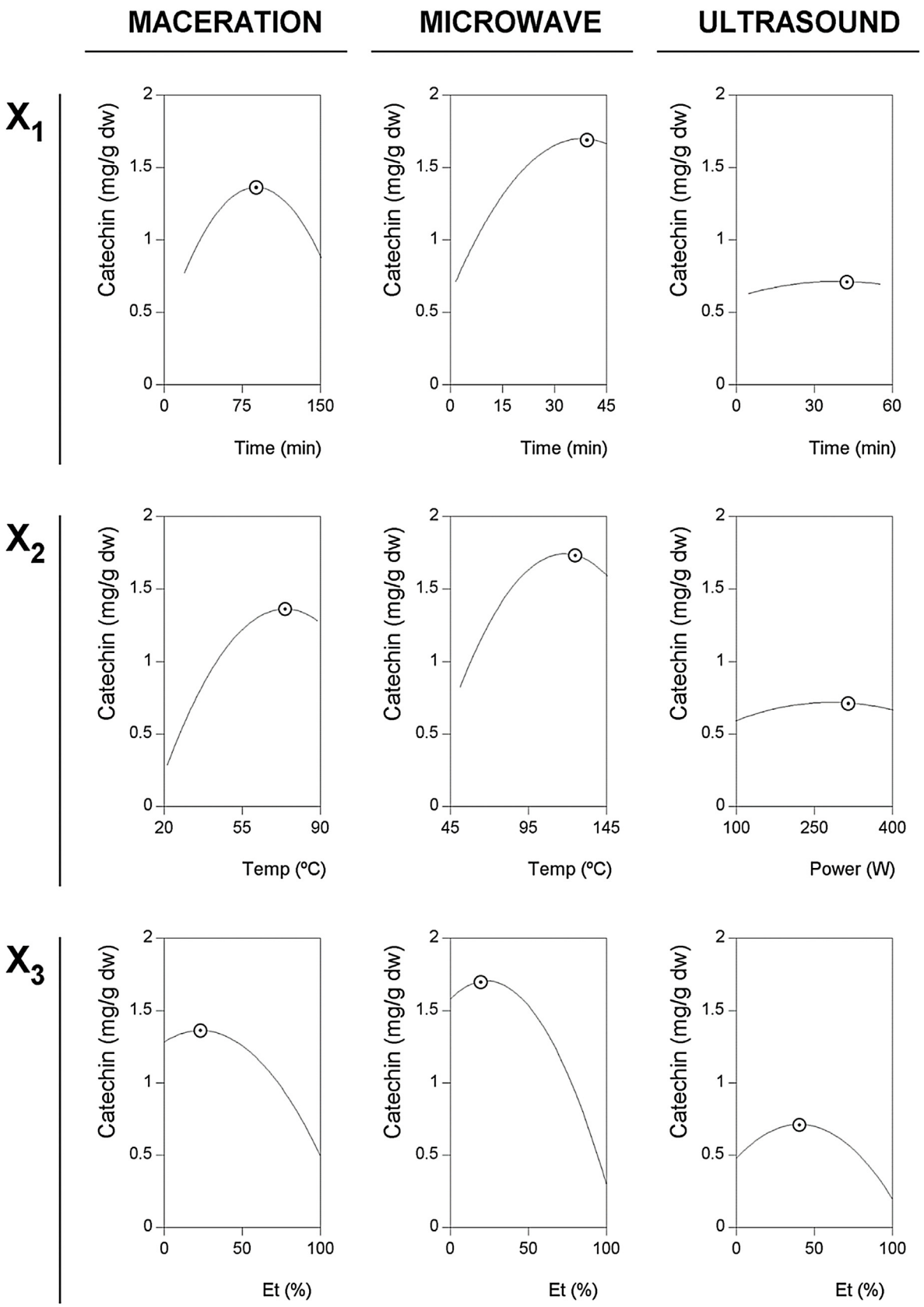

Fig. 3. Individual 2D responses of ME, MAE and UAE for each variable. Each graph shows a line and a dot. The line represents the response of the variable when the others are positioned at the optimal conditions found (third part of Table 4). The dots (⊙) presented alongside each line highlights the location of the optimum value. Lines and dots are generated by the theoretical second order polynomial models of Eqs. (2)–(6). Parametric fitting values obtained are presented in Table 3.

4. Conclusions

The extraction process for ME, MAE and UAE was successfully optimized by applying the RSM. The results showed that extraction time, temperature and ethanol content in the used water/ethanol mixtures have significant effects on the catechin extraction yield. ME and MAE were found to be the most effective methods capable of yielding 1.38 ± 0.1 and 1.70 ± 0.3 mg of catechin/g dw at their optimal extraction conditions. MAE was found to be faster and more effective in comparison with other studied techniques, but lower temperature was applied in ME with nearly identical extraction yields, which can be translated in economic benefits. The UAE was the less effective solution in terms of catechin extraction yield.

This work offers an overview through environmental compatible extraction processes, in which the valorisation of *A. unedo* fruits is performed by 'clean' technologies able to integrate a potential industrial sector in a sustainable approach (Boukroufa et al., 2015). The obtained results indicate the viability of using *A. unedo* fruits as a productive source of catechin by any of the assessed techniques and provide evidence of the advantages of maceration, microwave and ultrasound extraction techniques for their industrial production (Achat et al., 2012). In addition, the present work can reinforce the production of *A. unedo* fruits to serve as a source of bioactive compounds to be used as natural additives in functional foods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2016.10.050.

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